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CORRELATION OF TOLL-LIKE RECEPTOR 4 AND TUMOUR NECROSIS FACTOR ALFA WITH TYPE-2 DIABETES MELLITUS

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Abstract

Type 2 diabetes mellitus (T2DM) is a multifactorial chronic disease. It is the leading cause of premature death worldwide. Inflammatory cytokines have been reported with their potential enhancement to insulin resistance and increase the prevalence of T2DM. Therefore, an assessment of the immunological factors associated with T2DM could help in better monitoring and discovering new immunological treatments for T2DM. This study aimed to evaluate the levels of certain immunological parameters, tumour necrosis factor alfa (TNF-α) and toll-like receptor 4 (TLR4) as potential indicators for T2DM disease in a sample of the Iraqi population. A total of 90 subjects were enrolled in our study. These were chosen from three group categories: young T2DM patients, old T2DM patients, and healthy control. Both males and females were enrolled. The BMI and HbA1c were measured for all participants. Also, serum expression levels of TLR4 and TNF-α were evaluated for all participants by using the ELISA technique. Data were analysed using the statistical software SPSS 26. The results revealed the presence of significant differences between the three tested groups regarding BMI, HbA1c, and TLR4; the diabetic groups had the higher significant means in comparison with the healthy control group. In contrast, TNF-α did not show a significant difference among the participants. BMI had a significant correlation with HbA1c and TLR4. Also, there was a significant correlation between TLR4 and TNF-α, which ensures the immunological effect during T2DM. The findings revealed that TLR4 could be regarded as an early prediction biomarker for T2DM because it had a significant correlation with HbA1c among both young and old diabetic patients and increases as the HbA1c increases.

Keywords: BMI, Cytokines, Innate immunity, T2DM, TLR4, TNF-α, Toll-like receptor, tumour necrosis factor alfa, Type 2 diabetes.

1. Introduction

Diabetes mellitus (DM) is a chronic health concern that affects an immense percentage of the global community. Contemporary medicine has dedicated significant scientific efforts to address the rising incidence of the issue. Type 2 diabetes mellitus (T2DM) is a very intricate and multifactorial disease that requires more study to determine the best reliable treatments. With a high prevalence of diabetes, Iraq ranks as a top public health issue among Middle Eastern nations [1]. According to global trends regarding the incidence of diabetes mellitus, diabetes has become an epidemic issue in Iraq in the past decade, with a drastic rise of 115% in its incidence, from 19.58/1000 persons in 2000 to 42.27/1000 persons in 2015 [2]. T2DM is generally a more prevalent condition than type 1 diabetes, accounting for up to 90% of cases of DM. It was previously considered a disease affecting people aged forty years or older, but in the last two to three decades, T2DM has become more common among young children and adolescents [3].

Diabetes mellitus is a chronic metabolic illness that damages the heart, vasculature, eyes, kidneys, and nerves due to high blood glucose levels, according to the WHO. Over 90% of diabetes patients are T2DM and are characterized by insufficient insulin secretion from pancreatic islet β-cells, tissue resistance to insulin, and inadequate compensatory insulin response [4]. Hyperglycaemia results from insulin secretion failing to maintain glucose homeostasis as the illness progresses, which is the hallmark of T2DM [5].

Insulin resistance refers to the condition when an organism's cells fail to respond properly to normal insulin levels. Consequently, this impairs the ability of insulin to regulate glucose and lipid homeostasis. Such a condition would increase insulin secretion from beta cells to compensate for hyperinsulinemia. The prolonged period of this condition exhausts pancreatic beta cells and forces them to apoptosis. Also, insulin resistance promotes hepatic gluconeogenesis, interprets glucose uptake in the muscles, and induces adipose tissue lipolysis [6].

Insulin resistance may arise due to a combination of acquired and hereditary causes. Mutations and polymorphisms of insulin receptors, glucose transporters, and signalling proteins involved in insulin signal transduction are among the prevalent genetic abnormalities [7]. Obesity, physical inactivity, advanced glycation end products (AGE), excess free fatty acids (FFAs), psychological stress, smoking, alcohol use, or certain drugs are among the acquired causes of insulin resistance [8]. All these factors are associated with persistent low-grade inflammatory conditions and the production of inflammatory cytokines [9]. Inflammation is an important component linking insulin resistance with nutrient overload and increased visceral adipocyte mass [10]. During an insulin-sensitive state, the signalling cascade of insulin upon binding to its receptor results in the phosphorylation of tyrosine residues of the insulin receptor substrate-1 (IRS-1), ensuing in downstream insulin signalling. Nevertheless, during a condition of insulin resistance, inflammatory molecules stimulate multiple other serine kinases such as inhibitors of NFKB kinase subunit beta (IKK- β), ribosomal protein S6 kinase (S6K), PKC and glycogen synthase kinase 3β, and others. Activation of the later kinases causes insulin inhibition by phosphorylation of serine residues rather than tyrosine residues in the insulin signalling pathway [11].

Inflammatory cytokines were found to be elevated in T2DM patients. They can be generated by a variety of cell types and subsequently secreted into the circulation [12]. These cytokines elicit a tissue-specific immune response through their intricate interactions with specific target tissues. The Toll-like receptors recognize danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Activated TLR4 induces most proinflammatory cytokines that cause insulin resistance, which increases T2DM risk. TLR4 regulates innate immunity and is expressed in Macrophages, adipose tissue, pancreatic β-cells, airway epithelia, vascular endothelial cells, skeletal muscle, and smooth muscle cells [13]. TLR4 interacts with heat-shock proteins, fibronectin, fibrinogen, FFAs, and saturated fatty acids. This protein is also a vital receptor for Gram-negative bacteria's LPS [14]. Other cytokines, such as TNF-α and IL-6 are highly expressed in activated macrophages. TNF-α is a proinflammatory cytokine that aids in the healing process after tissue injury or infection and facilitates immune cells' migration towards the defective tissues [15]. TNF-α causes insulin resistance by blocking the phosphorylation of IRS-1 and Akt substrate 160 in the insulin signalling cascade pathways. The TNF-α is a potential marker that mediates the balance between insulin resistance and diabetes mellitus [16].

Diabetes has become an epidemic issue in Iraq, with a drastic recorded rise of 115% in its incidence from year 2000 to year 2015. Previous studies mentioned that T2DM and its complications are correlated with the parameters of innate immunity [17]. This has led to the hypothesis that this disease is dependent on the immune system [18]. Accordingly, this article focused on the role of TLR4 and TNF-α in the T2DM disease among different age categories to determine their correlation with this disease in a sample of the Iraqi population. The research started with the identification of the samples and performing the necessary formal individual and organizational agreements to acquire the required data. The samples have been clustered in three groups of age and subjected to tests and analysis.

2. Research Methodology

2.1. Problem formulation and development of research structure

This study included a collection of 90 blood samples from three group categories: old T2DM patients, young T2DM patients, and healthy control group. Their ages were segregated into groups of 40-80y, 10-35 and 10-65y, respectively. Each group consisted of 30 blood samples, and both sexes were enrolled. These patients' samples were collected randomly from Baghdad medical clinics. In contrast, healthy control samples were obtained from medical staff, colleagues, and random volunteers who did not have diabetes or any other health issue. The donors consented to participate in this study after receiving a comprehensive elucidation regarding the research's objectives and the required tests. Physicians have followed the American Diabetes criteria to diagnose T2DM patients, which consider fasting blood glucose levels over 126 mg/dl, or 8 mmol/l. The current study enrolled patients who had HbA1c $\geq$ 8 mmol/l. The excluded criteria were pregnancy, liver disease, intoxication, chronic renal disease, thyroid disorders, or any endocrine disorder. Figure 1 illustrates the study flowchart.

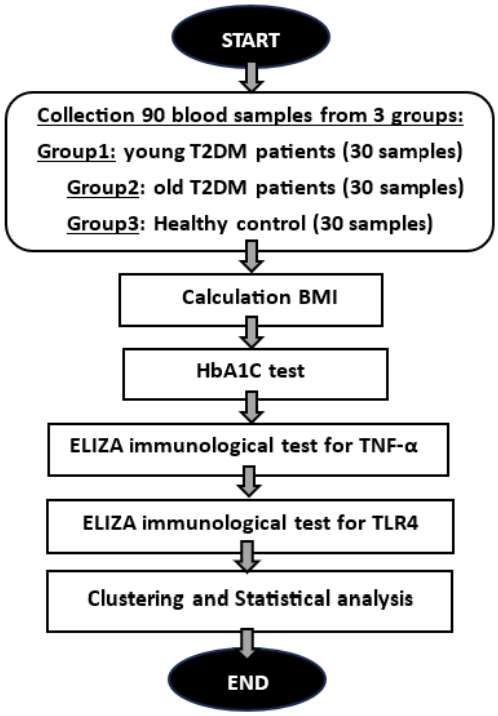

Fig. 1. Methodology flowchart of the study.

2.2. Samples collection

From each participant, five millilitres of peripheral venous blood were extracted. They were separated into two tubes in accordance with the needs of the research. The first tube was the EDTA tube, which contained two millilitres of blood and was preserved at -20 ºC until use in the HbA1c test. The remaining blood samples were added to the coagulation activator gel tube, kept for a few minutes at 20 to 25 °C, and then centrifugated at 3000 rpm/minute for fifteen minutes to separate the serum from the clot and depress. Then, the upper serum was withdrawn and kept in a clean test tube at -20 ºC till it was used in an ELISA immunological test for TLR4 and TNF-α.

2.3. HbA1c test and BMI calculation

The HbA1c measuring was done following the manufacturing instructions of the kit-providing company (Ichroma TM HbA1c - glycosylated haemoglobin/ Biotech – Spain). The BMI was calculated according to the equation:

$$\text{BMI } \left(\frac{kg}{m^2}\right) = \frac{mass\ (\text{kg})}{height^2 (\text{m}^2)} \quad (1)$$

2.4. ELISA immunological tests for TLR4 and TNF-α

The concentration of TLR4 and TNF-α was detected by using the ELISA technique. Each test was estimated separately by following the instructions of the kits provided company: Human Tumour necrosis factor-α (TNF-α) ELISA Kit (INNOVA

Biotech co-limited/ China), and Human Toll-Like Receptor 4 (TLR4) ELISA Kit (INNOVA Biotech co-limited/ China). The basis of the previous kits depended on enzyme-linked immunosorbent assay (ELISA) reagent. About 10% of the serum samples were used for duplicate measurements.

2.5. Statistical analysis

The statistical analysis of the results was conducted using SPSS 26 (SPSS Inc., USA). Means ± standard deviations were used to express the data. The significance was determined according to the p-value. If p-value ≤ 0.05* is typically significant. If p-value is ≤ 0.001, then there would be a one-in-1,000 chance of observing results at least as extreme. To analyse the differences between the three groups that have been enrolled in our study, one-way ANOVA and Chi-square tests have been used. Duncan's multiple range comparison (DMRT) test was done and gave different letters to the distinct means. ROC test (receiver operating characteristic curve) was done to determine the sensitivity and specificity of TLR4 and TNF-α.

Sensitivity means "positivity in disease". It referred to the proportion of subjects who had the target condition (reference standard positive) and gave positive test results. Specificity means "negativity in health" and refers to the proportion of subjects without the target condition who gave negative test results.

The Pearson correlation test was done to discover the correlations between the parameters that were included in our study according to the value of r (correlation coefficient) and the p-value of the test. Statistically,

- If the p-value ≤ 0.001, that means there is a highly significant result,
- If the p-value ≤ 0.05, that means there are significant results.
- If p-value > 0.05 that means the results are non-significant.

2.6. Ethical approval

The current study was conducted after obtaining the approval of the local ethical committee of the Genetic Engineering and Biotechnology Institute/ University of Baghdad/ Iraq, with reference number (EC/1310-F) on 15/5/2022.

3. Results and Discussion

The current study included 3 groups: young T2DM patients, old T2DM patients, and healthy control group. The age distribution, BMI, HbA1c test, TNF-α test, and TLR4 test results for these groups are illustrated in Table 1.

The BMI was calculated for these groups according to the data of each participant. The results revealed that there was a high significant difference (p-value = 0.0001) between the three groups. The BMI level was higher in the diabetic groups in comparison with the healthy control group. The old T2DM patients had the highest BMI mean (34.216 ± 5.703 kg/m^2), followed by the young T2DM patients with a BMI mean of 31.434 ± 5.457 kg/m^2, while the healthy control group had the lowest BMI level (24.554 ± 1.5 kg/m^2). The definition of body mass index (BMI) is weight in kilograms divided by square height in meters. The BMI classification has four categories: underweight, less than 18.5 kg/m^2, normal weight between 18.5 and 25.0 kg/m^2, overweight 25.0 to 30.0 kg/m^2, and obese, over 30.0

kg/m^2 [19]. According to that classification, our results recorded diabetic patients as overweight due to having a BMI lower than 30 kg/m^2. The global incidence of obesity and type 2 diabetes continues to rise, affecting a significant proportion of people because of their busy schedules and sedentary lifestyles [20].

Table 1. Distribution of age, BMI, HbA1c, TNF-α, and TLR4 among the three groups.

Parameter	Group	No.	Mean	Std. Deviation	Std. Error	P-value
Age (years)	Young T2DM patients	30	23.133c	7.789	1.4221	
	Old T2DM patients	30	57.700a	8.762	1.5997	0.0001
	Control	30	36.733b	16.98	3.0995	
BMI (kg/m^2)	Young T2DM patients	30	31.435b	5.457	.9964	
	Old T2DM patients	30	34.216a	5.703	1.0412	0.0001
	Control	30	24.554c	1.500	.2739	
HbA1c	Young T2DM patients	30	9.51b	0.999	.1824	
	Old T2DM patients	30	10.48a	1.838	.3357	0.0001
	Control	30	5.07c	0.355	.0648	
TNF-α (pg/ml)	Young T2DM patients	30	75.97a	12.15	2.2183	
	Old T2DM patients	30	67.12a	27.87	5.0884	0.131
	Control	30	63.97a	27.44	5.0095	
TLR4 (pg/ml)	Young T2DM patients	30	869.8a	223.75	40.851	
	Old T2DM patients	30	730.7b	190.08	34.704	0.0001
	Control	30	614.4c	211.09	38.540	

Previous studies mentioned that excessive body fat buildup may lead to the development of type 2 diabetes, and the likelihood of developing T2DM rises proportionally with an increase in body mass index. The connection between obesity and T2DM involves intricate cellular and physiological processes. It includes changes in beta cell function, adipose tissue biology, and insulin resistance in multiple organs, all of which are influenced by excess body fat. However, effective weight loss can improve and potentially reverse these effects [21].

The HbA1c results showed that there was a highly significant difference between the three groups (p-value = 0.0001). The old T2DM patients had the highest HbA1c mean of 10.4867 ± 1.84, followed by the young T2DM patients with HbA1c mean (9.51 ± 1.0), while the healthy control group had the normal HbA1c level of 5.069 ± 0.36. This result is constituent with previous studies that indicated that age was a contributing factor to elevated HbA1c values. They also mentioned that HbA1c concentrations are influenced by age and gender disparities, and the elevated HbA1c values indicate the involvement of non-glycaemic causes. The prevalence of diabetes and prediabetes rises globally as people age, and glycemia fluctuates with age [22]. HbA1c helps diagnose and track diabetes. HbA1c provides information about the mean glucose concentration for 100 – 120 days before the test date. The HbA1c levels at 6.5% were considered as diagnostic criteria for diabetes. HbA1c may rise owing to extended glucose exposure due to reduced macrophage activity [23]. Age-related cellular damage in erythrocytes due to diminished membrane lipids, enzyme activity, and cell fragility may accelerate glycation. In addition to renal impairment caused by ageing, B12, iron, and spleen

malfunction are linked to higher HbA1c levels without hyperglycaemia. Thus, an older adult's HbA1c values may not accurately represent mean glucose[[24].

ELIZA tests were done to measure the levels of TNF-α and TLR4 among the three groups. The results of Table 2 revealed that there was a highly significant difference between the groups for TLR4 with p-value = 0.0001, as shown in Fig. 2. The young T2DM patients had the highest means of TLR4 (869.8 ± 223.75 pg/ml), followed by the old T2DM patients with a mean (730.7 ± 190.08 pg/ml), while the healthy control group had the lowest level of TLR4 (614.4 ± 211.1 pg/ml). On the other hand, no significant differences had been recorded between the three groups regarding the TNF-α, where the p-value = 0.131, as shown in Fig. 3.

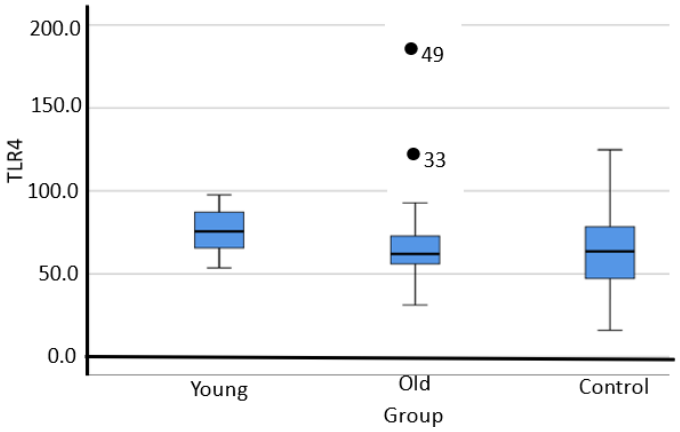

Fig. 2. The boxplot of TLR4 distribution among the three tested groups (young T2DM patients, old T2DM patients, and the healthy control group.

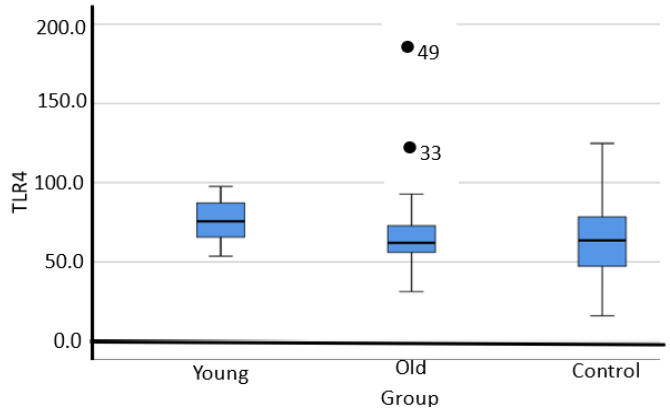

Fig. 3. The boxplot of TNF-α distribution among the three tested groups (young T2DM patients, old T2DM patients, and the healthy control group.

The current study also investigated the Distribution of age, BMI, HbA1c, TNF-α, and TLR4 among gender as illustrated in Table 2. The results revealed that there were no significant differences between males and females regarding age, BMI, HbA1c, TNF-α, and TLR4. The p-value > 0.05.

Table 2. Distribution of age, BMI, HbA1c, TNF-α, and TLR4 among gender.

Parameter	Gender	No.	Mean	Std. Deviation	Std. Error	p-value
Age	**male**	48	36.54	17.05	2.46	0.148
	female	42	42.21	19.84	3.06	
BMI	**male**	48	30.17	6.11	0.88	0.872
	female	42	29.96	6.25	0.96	
HbA1c	**male**	48	8.13	2.65	0.38	0.393
	female	42	8.61	2.68	0.41	
TNF-α	**male**	48	67.52	19.46	2.81	0.526
	female	42	70.74	28.34	4.37	
TLR4	**male**	48	724.44	196.21	28.32	0.547
	female	42	754.13	267.93	41.34	

According to our results, there are no significant variations in HbA1c values between men and females. This finding was in accordance with Taha et al. [25] conclusion that there were no gender-related variations in T2DM. While another research conducted by Hovestadt et al. [26] studied 2455 adolescents and healthy children, they reported increasing levels of HbA1c in males. This increase was positively correlated with age. They concluded that there were post-childhood gender differences in healthy individuals.

The ROC test has been done to measure the sensitivity and specificity of the TLR4 ELIZA test, and the results revealed that this test gave a highly significant result with a p-value (0.0001). The sensitivity of this test was 65.5%, and the specificity was 73%. The result of this test was reliable and had a good explanation due to the AUC (0.72), which is explained as a good result (Table 3 and Fig. 4). So, we can rely on the result that TLR4 increases in T2DM patients in young and old patients in comparison with healthy individuals.

The ROC test has been done on TNF-α to determine this test's sensitivity and specificity. The result was a high significant p-value = 0.0001, but this test fails to express the diabetic condition due to the low level of AUC (0.555). The sensitivity and specificity were 40.7% and 64.4%, respectively (Table 4 and Fig. 5).

Table 3. The ROC curve for the TLR4.

Parameter	Result
Area Under the Curve (AUC) for TLR4	0.720
Explanation	Good
Standard error	0.006
Asymptotic Sig (*P* value)	0.0001
The best Cut off	746.7
Sensitivity %	65.5
Specificity %	73.0
Asymptotic 95% Confidence-Interval- Lower Bound	0.708
Asymptotic 95% Confidence-Interval-Upper Bound	0.732

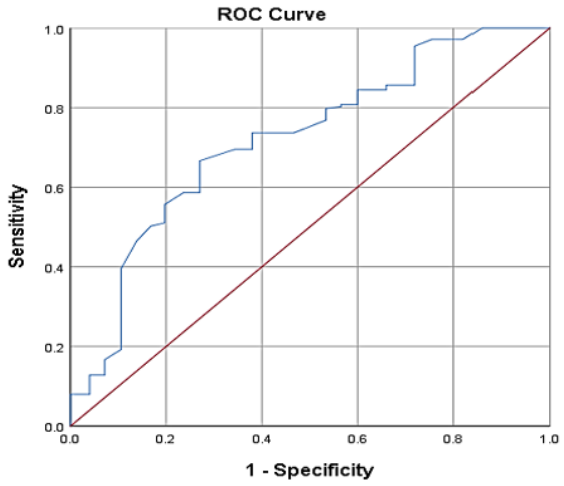

Fig. 4. The boxplot of TNF-α distribution among the three tested groups (young T2DM patients, old T2DM patients, and the healthy control group.

Table 4. The ROC curve for the TNF-α.

Parameter	Result
Area Under the Curve (AUC) for TNF-α	0.555
Explanation	Fail
Std. error	0.007
Asymptotic Sig (p-value)	0.0001
The best Cut off	70.0
Sensitivity %	40.7
Specificity %	64.4
Asymptotic 95% Confidence-Interval- Lower Bound	0.542
Asymptotic 95% Confidence-Interval-Upper Bound	0.569

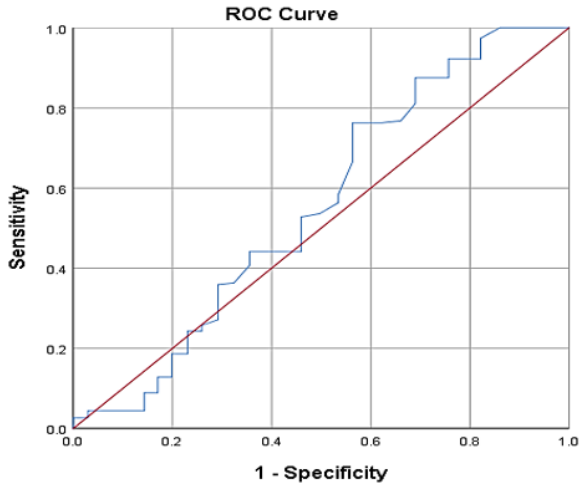

Fig. 5. The TNF-α versus ROC sensitivity curve.

The correlation test was done to explore the relationship between the different parameters that were included in our study. The results are illustrated in Table 5. There were significant correlations between BMI and each of (age and HbA1c) with correlation coefficient (r): 0.318 and 0.665, respectively. High significant correlations had been recorded between TLR4 and each of HbA1c and TNF-α, with r: 0.355 and 0.631, respectively. Also, a significant correlation between TLR4 and BMI was recorded with r = 0.240. No significant correlation was recorded between the other parameters.

Table 5. The ROC curve for the TNF-α.

Parameter	Statistic	Age	BMI	HbA1C	TNF-α	TLR4
Age	Pearson Correlation	1	0.318**	0.189	-.172	-0.162
	Sig. (2-tailed)		0.002	0.075	0.105	0.126
	No.	90	90	90	90	90
BMI	Pearson Correlation	0.318**	1	0.665**	0.152	0.240*
	Sig. (2-tailed)	0.002		0.000	0.153	0.023
	No.	90	90	90	90	90
HbA1C	Pearson Correlation	0.189	0.665**	1	0.192	0.355**
	Sig. (2-tailed)	0.075	0.000		0.069	0.001
	No.	90	90	90	90	90
TNF-α	Pearson Correlation	-0.172	.152	0.192	1	0.631**
	Sig. (2-tailed)	0.105	.153	0.069		0.000
	No.	90	90	90	90	90
TLR4	Pearson Correlation	-0.162	0.240*	0.355**	0.631**	1.0
	Sig. (2-tailed)	0.126	0.023	0.001	0.000	
	No.	90	90	90	90	90

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

The results revealed that there was a positive correlation between BMI and age among all participants. This result is constituent with that conducted on the Saudi population, who confirmed that there was a significant increase in obesity prevalence with age in both genders [27]. The results revealed that there was a highly significant positive correlation between TLR4 and HbA1c. The results of the TLR4 ROC test gave an accepted value of AUC and moderate sensitivity and specificity. These results confirmed the correlation between TLR4 and T2DM disease among different age categories. This finding agreed with a previous study that demonstrated that the TLR4 level was higher in diabetic patients than in the healthy control group [28]. TLR4 are cytokines that represent the first line of defence, regulate immunity by identification of pathogen-association molecular patterns, and activate the immune response genes [29]. Prior studies have identified TLR4 as a potentially significant factor that regulates susceptibility to T2DM. The genetic alteration in TLR4 has been linked with an increasing prevalence of T2DM [30]. Thus, it's important to test the levels of TLR4 in different ethnic groups to evaluate their effectiveness among different populations.

In addition, the presence of a significant positive correlation between TLR4 and BMI is in accordance with a prior study by [31] that mentioned that the TLR4 expression increased with obesity. Previous studies mentioned that the TLR4 expression level was elevated in T2DM patients and had an important role in diet-induced obesity and related resistance to insulin [32]. Another previous study also agreed with our results regarding the correlation between TLR4 and T2DM, and they confirmed the implication of TLR4 in insulin resistance and T2DM disease [33].

The present study revealed that there was a positive significant correlation between TLR4 and TNF-α. This finding is constituent by the fact that the activation of TLR4 with long fatty acids and lipopolysaccharides activates the MyD88-dependent pathway, and this leads to NF-kB activation, then increases the expression of inflammatory regulatory genes like TNF-α, interleukin-1, interleukin-6, and MCP-1 [34]. On the other hand, our results did not record a positive correlation between TNF-α and HbA1c. This result is not consistent with preclinical studies that mentioned TNF-α as a cytokine that induces insulin resistance. TNF-α inhibitors could reduce glycemia, fasting glucose level, and incidence of diabetes [35]. The obtained result may be interpreted by the limited sample number that has been enrolled in this study. Also, various environmental risk factors are encountered by populations in various geographic locations, leading to the formation of distinct gene-environment interactions [36]. This may change the way that various populations' genes affect T2DM, leading to inconsistent findings [37]. Similar results had been recorded in a previous study regarding another cytokine, interleukin 18, in the same groups [38], which may be interpreted due to the presence of genetic variation affecting the cytokine output among different populations [39].

4. Conclusions

Diabetes has become an epidemic issue in Iraq, with a drastic rise of 115% in its incidence within the period of 2000 to 2015. TLR4 is correlated with type 2 diabetes mellitus and could be utilized as a biomarker for this disease. The best cutoff value for the TLR4 ELIZA test was 746.67 pg/ml. The TLR4 had a positive correlation with HbA1C and BMI, which could be an indicator for them. Gender differences did not show a significant correlation with each of BMI, HbA1C, TNF-α and TLR4. TNF-α did not show a significant correlation with this disease. More genetic studies should be done to explore the exact role of TNF-α in diabetes and discover mutations or polymorphisms that could exist in the Iraqi population.

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